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BACTERIAL TRANSPORT THROUGH HOMOGENEOUS SOIL

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Summary—The transport of microorganisms in soils is of major importance for bioremediation of *subsurface polluted zones and for pollution of groundwater with pathogens. A procedure for evaluating* the relative mobility and recovery of bacteria in the soil matrix was developed. In the method devised, movement of bacteria along the walls of the column of soil and channeling were prevented. Changes in population size during the test period were minimal because temperatures of 2–5°C were maintained and predators and parasites were eliminated by ^{60}Co irradiation. The 19 strains of bacteria tested had markedly different degrees of transport. From 0.01 to 15% of the added cells passed through a 5-cm long column of Kendaia loam with four pore volumes of water and from 4.3% to essentially all of the added bacteria were recovered. The marked differences in the mobilities of the various bacteria and the high recoveries of most of the isolates suggested that the procedure developed is a useful means for selecting bacteria according to their mobilities in soils, aquifer materials, and other porous media.

INTRODUCTION

The bioremediation of underground waste-disposal sites by the use of introduced bacteria requires that the microorganisms move from the point of their introduction to the site of contamination. Such inoculation is necessary if microorganisms degrading the chemical contaminants are not present in the hazardous-waste site or adjacent groundwaters. Should the introduced bacteria fail to survive or move through the unsaturated zone or aquifer, bioremediation will not occur. It has thus been reported that a *p*-nitrophenol-degrading bacterium added to the soil surface failed to mineralize much of the nitro compound unless it was mixed into the soil (Goldstein *et al.*, 1985).

Considerable attention has been given to the mobility of bacteria and other microorganisms in soil and subsurface materials. These studies were conducted primarily because of concern with the dissemination of pathogens from land spreading operations, groundwater recharge or the disposal of manure or municipal sludge (Gerba *et al.*, 1975; Brown *et al.*, 1979; Bell and Bole, 1978). Several studies have shown poor mobility of the investigated species of bacteria through soil (Bitton *et al.*, 1974; Wollum and Cassel, 1978; Madsen and Alexander, 1982). However, considerable movement of some bacteria was observed in field studies (Schaub and Sorber, 1977; Viraraghavan, 1978), and rainfall or artificial additions of water enhance the transport of viruses through soil (Duboise *et al.*, 1976; Gerba and Lance, 1978; Sobsey *et al.*, 1980). It is unclear whether the movement of bacteria that has been observed occurred through the soil matrix or through the macropores or channels that afford the organisms a relatively unhindered passage (Hagedorn *et al.*, 1981; Rahe *et al.*, 1978). Both adsorption (Hattori and Hattori, 1976; Marshall, 1980) and mechanical filtration (Pekdeger and Matthess, 1983; Smith *et al.*, 1985) of bacterial cells have been suggested as mechanisms for their retention in soils. Soil structure and the velocity of water flow appear to be major determinants of the movement of bacteria (Smith *et al.*, 1985; Harvey *et al.*, 1989). The use of soil columns for studies of bacterial transport has been suggested to give rise to misleading results (Bitton *et al.*, 1979).

Our objectives were to develop a reproducible procedure that would yield consistent measurements of relative mobility of bacteria in soil by avoiding uncontrolled variations in bacterial behavior and to relate transport to efficiency of recovery and adsorption of the cells. In the procedure that was developed, flow through macropores did not occur.

MATERIALS AND METHODS

Bacteria able to degrade benzene, chlorobenzene or toluene were isolated by enrichment culture in solutions containing 100 mg of the organic compound, 1.6 g K_2HPO_4, 0.4 g KH_2PO_4, 0.5 g $(NH_4)_2SO_4$, 25 mg $CaSO_4 \cdot 2H_2O$, 0.2 g $MgSO_4 \cdot 7H_2O$ and 2.3 mg $FeSO_4 \cdot 7H_2O$ l^{-1} deionized water. The pH was adjusted to 7.0. Each enrichment was transferred at least four times into fresh medium of the same composition before plating the bacteria on enrichment medium supplemented with 1.5% agar. Biodegradation was determined by spectrophotometric measurement of the loss of UV absorbance of the added organic compound. To obtain bacteria by a method that presumably favored mobile organisms, diesel-fuel contaminated soil, Kendaia loam and liquid from the primary settling tank of the Ithaca, N.Y. sewage treatment plant were placed on top of the soil column

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described below. The soil column was then leached with 4 pore vol of water. Bacteria that passed through the soil were isolated on Trypticase-soy agar (BBL Microbiology Systems, Cockeysville, Md). *Bacillus* sp. CU 4519 was obtained from S. A. Zahler, and *Arthrobacter* sp. Lula D from an aquifer was provided by J. L. Sinclair, both from Cornell University.

The bacteria were grown in Trypticase-soy broth at 30°C for 24–48 h, the cells were harvested by centrifugation and washed twice, and the organisms were suspended and diluted in 0.9% NaCl solution. The cell suspensions were cooled to 3.5 ± 1.5°C for 1 h prior to their addition to columns of soil. Bacterial counts were made on Trypticase-soy agar using triplicate samples from the effluent of each soil column and triplicate plates per dilution. The plates were kept at 30°C for 24–48 h.

Kendaia loam (37.1% sand, 40.4% silt, 22.5% clay, pH 6.4, cation-exchange capacity 26.5 cmol kg^{-1}) was air-dried, ground, sieved (< 2 mm) and then sterilized by ^{60}Co irradiation (2.5 Mrad). Aseptic conditions were maintained during the experiments. The primary clay species in this soil is vermiculitic, but small amounts of mica and chlorite are also present. The soil was packed in 600-ml Buchner funnels, 10 cm dia, each containing a fritted-glass disc with pore sizes of 40–60 μm. Tests had indicated that the pore sizes in the disc permitted passage of essentially only bacteria and water-dispersible clay, and the rate of flow during the experiments remained constant for the 4 pore vol of water passed through the columns, indicating that clogging did not occur. The funnel was attached to a 500-ml Erlenmeyer flask fitted with a sidearm containing Hg in a bulb to control the flow rate. The walls of the funnel were coated with a thin layer of sterile petrolatum added in liquid form at >60°C. The petrolatum sealed the interface between the funnel walls and the soil. The soil was pressed down with a fitted brass compacting plate to a depth of 5 cm to give a bulk density of 1.080 ± 0.062 g cm^{-3} and a porosity of 59.2%.

The soil columns and sterile deionized water to be used for mobility tests were cooled to 3.5 ± 1.5°C, the temperature at which experiments were conducted. The columns were moistened from below by connecting a bottle of water to the stem of the Buchner funnel. Sterile deionized water was added at a rate of *ca* 10 cm h^{-1} until the water level was *ca* 2 cm above the soil surface. The water was then drained to the surface of the soil column, and a circular mound with a radius of 1 cm of dry sterile soil was applied to the center of the soil surface. A 1.0-ml inoculum of 1×10^8 cells was placed in the center of that mound. An additional 1-cm layer of sterile soil was added to the top of the column, thereby covering the inoculated mound and making the column surface approximately level. Deionized water was added to the soil surface after inoculation. Physical disturbance of the surface of the soil column was minimized by pouring the water onto Al strips placed above the soil surface. A total of 4 pore vol of effluent was collected. The rate of water flow through the column was maintained at *ca* 0.8 pore vol h^{-1}.

The distribution of microorganisms in the column at the end of the test and possible transport of bacteria along the walls of the column were determined as follows. After the column was leached with 4 pore vol and allowed to drain, five vertical cores (5 cm in length) were taken from the soil column using a 5-ml syringe (1.4 cm i.d., 1.7 cm o.d.) from which the luer end was cut. A core taken from the center of the column was divided into sections taken from depths of 0.0 to 1.7, 1.7 to 3.4 and 3.4 to 5.0 cm. Cores were also taken at distances of 2 and 4 cm between the core center and the column center. Two cores were taken at each such distance at diametrically opposed points per column. Determinations of bacterial distribution were conducted with duplicate columns. The total number of bacteria in each core and core section was then determined.

Determinations were made of the proportion of the cells added to the soil that appeared in the effluent (percentage transported) and the proportion that could be recovered from both the effluent and the soil column at the end of the test period (percentage recovery). Triplicate columns were inoculated. The bacteria in the effluents and those remaining in the soil of two of the three inoculated columns were counted after passage of 4 pore vol of deionized water. For this purpose, the soil removed from the column was shaken with deionized water (1:2.5) for 5 min on a rotary shaker operating at 120 rev min^{-1}, and bacterial counts were made after the larger particles were allowed to settle for 5 min.

Adsorption of bacteria to soil was determined by a procedure based on the difference in gravity sedimentation rates of the bacterial cells and sand, silt and clay size fractions of the soil. At an experimental temperature of 3°C, the time required for the size fraction ⩾2 μm of the soil to settle below an 8-mm depth is predicted by Stokes' law (Jackson, 1974) to be 65 min. To determine the amount of water-dispersible clay (< 2 μm) that remained suspended with bacteria at the sampling depth, a 20-g portion of Kendaia loam was shaken for 1 h with 100 ml of deionized water. The suspension was then diluted to 1 litre with deionized water and allowed to settle for 4.5 h at 21 ± 1°C. A 25 ml portion was then taken from the 5-cm depth of a 1-litre graduated cylinder and dried overnight at 105°C. The water-dispersible clay constituted 2.6% of the total soil or 12% of the total clay content.

To measure bacterial adsorption at 3°C, 20-g samples of Kendaia loam and *ca* 10^8 cells were shaken for 1 h with 100 ml of deionized water. The suspension was allowed to settle for 65 min in a sterile 250-ml graduated cylinder. Determinations were made of the number of bacteria at the 8 mm depth of the soil suspension (S_t) and of a control (C_t) to which no soil was added. The experimental and control suspensions were again thoroughly mixed, and the numbers of bacteria in the soil suspension (S_i) and the control (C_i) were determined. The drop plate method was used for bacterial counts (Reed and Reed, 1948).

The data were expressed as adsorption coefficients (K_d) where

$$K_d = F/D\,(C_i - F)^{-1} \qquad (1)$$

F is the number of bacteria adsorbed ml^{-1}, D is the concentration of soil in the suspension, and the value

of D is 0.2 (20 g 100 ml^{-1}). Lower and upper bounds for K_d were calculated. The lower-bound adsorption value assumes that bacteria retained by clay particles of equal or smaller size are not considered to be sorbed, and the upper-bound adsorption value considers all bacteria–clay interactions as adsorption.

To determine F, the equation takes into account bacterial settling and the interaction between bacteria and water-dispersible clay. The lower bound of adsorption is expressed by the following equation.

$$F_{low} = S_t - (S_s \times C_t/C_s) \quad (2)$$

The upper-bound of adsorption is expressed by the following equation,

$$F_{up} = [S_t - (S_s\ C_t/C_s)]\ (1 - W_d \times C_t/C_s)^{-1} \quad (3)$$

where W_d is the water-dispersible fraction of the clay (0.12).

RESULTS

Bacteria were obtained from Kendaia loam that were able to grow using benzene, chlorobenzene or toluene as sole source of C and energy. They were identified by standard bacteriological techniques as strains of *Pseudomonas*, *Achromobacter*, *Bacillus* and *Enterobacter*. The isolates able to degrade benzene were designated *Pseudomonas* Ben1 and *Achromobacter* Ben2, those degrading chlorobenzene were designated *Pseudomonas* CB1, *Bacillus* CB2 and *Bacillus* CB3 and the bacteria growing on toluene were designated *Enterobacter* Tol1, *Enterobacter* Tol2, *Enterobacter* Tol3 and *Pseudomonas* Tol4. The bacteria obtained from diesel fuel-contaminated soil were designated *Achromobacter* DF1, *Pseudomonas* DF2 and *Flavobacterium* DF3, those from the sewage treatment plant were designated *Enterobacter* strains IS1 and IS2 and those from Kendaia loam as *Enterobacter* KL1 and Pseudomonas KL2 and KL3. The last three isolates were not selected because of their ability to metabolize aromatic compounds.

Pseudomonas KL2 was used to study the direction of movement of bacteria through the soil column.

Table 1. Distribution of *Pseudomonas* KL2 in columns of soil after passage of 4 pore vol of water

Sample location	Sample depth	Cell no. (10^3 g^{-1}) Column 1	Column 2
Center of column	0.0–1.7	980	2200
	1.7–3.4	1400	730
	3.4–5.0	720	690
Left, 1.3–2.7 cm*	0–5.0	79	21
Right, 1.3–2.7 cm*	0–5.0	14	0.75
Left, 3.3–4.7 cm*	0–5.0	0.75	0.01
Right, 3.3–4.7 cm*	0–5.0	0.09	<0.01

*Lateral distance from the center of the column and left or right of center core.

The distribution of bacteria in the column after leaching showed that the direction of bacterial transport was chiefly downwards (Table 1). The presaturation of the soil columns resulted in only vertical mass water flow, and the bacteria thus moved with the water. Because little horizontal movement was evident and very few bacteria reached the walls, movement of cells along the column wall did not appear to contribute to measurements of the transport of bacteria.

In the studies of bacterial transport, the numbers of colony-forming units (cfu) on replicate plates from triplicate columns of soil agreed within a factor of ±3, except for *Bacillus* CU4519, *Pseudomonas* CB1 and *Bacillus* CB3, for which fewer than 10^5 of the 10^8 cells added to the top of the soil were transported and the agreement among replicate counts was poor (Table 2). In tests of the numbers of added bacteria that were recovered, replicate counts of the number of cfu agreed within a factor of ±2. Marked differences in the fraction of the added bacteria that moved through the soil were evident among the various species (Table 2). For *Enterobacter* IS2, 15% of the cells were transported, whereas only 0.01% of the added cells of *Bacillus* CB3 were found in the effluent as viable bacteria. No consistent pattern in mobility was evident among strains within a genus; thus, >5% of the cells of two strains of *Enterobacter* and three strains of *Pseudomonas* were transported as compared to <1% of three other strains of each of

Table 2 The numbers and percentages of bacteria transported and recovered

Bacterium	Bacteria transported (10^8 cfu)	Bacteria transported* (%)	Bacteria recovered* (%)
Enterobacter IS2	25–31	15 ± 2	61 ± 6
Enterobacter IS1	26–50	13 ± 3	60 ± 13
Pseudomonas KL2	22–32	8.2 ± 1	46 ± 6
Arthrobacter Lula D	14–26	7.7 ± 1.9	39 ± 4
Pseudomonas DF2	13–24	6.9 ± 0.4	53 ± 12
Achromobacter Ben2	6.4–8.5	6.8 ± 0.8	107 ± 13
Pseudomonas Ben1	5.0–5.8	5.9 ± 0.4	71 ± 4
Bacillus CB2	14–30	4.1 ± 1.2	104 ± 20
Achromobacter DF1	2.4–4.0	3.9 ± 0.8	39 ± 4
Enterobacter KL1	1.6–2.6	2.2 ± 0.4	86 ± 7
Pseudomonas KL3	2.3–4.4	0.9 ± 0.3	48 ± 3
Enterobacter Tol2	2.0–3.0	0.9 ± 0.1	14 ± 1
Enterobacter Tol3	1.3–1.5	0.9 ± 0.1	64 ± 1
Pseudomonas Tol4	0.49–1.1	0.3 ± 0.1	6.5 ± 1.5
Enterobacter Tol1	0.60–1.5	0.2 ± 0.1	34 ± 1
Pseudomonas CB1	0.15–0.48	0.2 ± 0.1	25 ± 5
Bacillus CU4519	0–0.29	0.1 ± 0.1	9.4 ± 1.3
Flavobacterium DF3	0.12–0.22	0.1 ± 0.01	5.4 ± 1.5
Bacillus CB3	0.0053–0.016	0.01 ± 0.00	4.3 ± 0.4

*Mean ± standard deviation

the same two genera. The benzene degraders (strains Ben1 and Ben2) moved to a greater extent than the isolates able to use chlorobenzene (strains CB1, CB2 and CB3) or toluene (Tol1, Tol2, Tol3 and Tol4). Of the eight isolates selected for their presumed greater mobility, most were relatively mobile. More than 2% of the cells of six strains (*Enterobacter* IS2, *Enterobacter* IS1, *Pseudomonas* KL2, *Pseudomonas* DF2, *Achromobacter* DF1 and *Enterobacter* (KL1) were transported through the soil, whereas the percentages were lower for *Pseudomonas* KL3 and *Flavobacterium* DF3. For the bacterium originally obtained from an aquifer (*Arthrobacter* Lula D), 7.7% of the cells moved through the soil.

The proportions of the added cells that were recovered in the soil and the effluent varied from essentially 100% for *Achromobacter* Ben2 and *Bacillus* CB2 to 4.3% for *Bacillus* CB3 (Table 2). Although some strains of a genus showed high recoveries, far lower recoveries were found among other strains of the same genus. The fact that the recoveries of only 5 of the 19 strains were below 25% indicates that a high percentage of the cells of many strains did not lose viability and were not strongly sorbed to soil particles. A comparison of recovery percentages and the transport percentages indicates that many of the viable cells were retained in the soil column. A regression of percentage of cells transported on percentage of bacteria recovered gave an F value of 5.08 (significant at $P = 0.05$). The data show that the recoveries exceeded 50% for 7 of the 10 isolates for which >2% of the cells were transported, but such high recoveries were found for only 1 of the 9 isolates for which <1% of the cells were transported. This is not surprising since, if it is assumed that low recovery results from sorbed cells not re-entering the stream of flowing water or their loss of viability, strains with many cells strongly sorbed or inactivated are not likely to have many viable cells transported.

The K_d values of the bacteria ranged from 0.0 for *Enterobacter* IS1 to infinity (upper bound of K_d) for *Pseudomonas* Tol4 (Table 3). The value of infinity reflects adsorption of all the cells. A comparison of the mean values between the lower- and higher-bounds of K_d values with the transport percentages shows that 8 of 10 bacteria for which >2% of the cells were transported had mean K_d values <10.0, whereas 8 of 9 bacteria for which <1% of the cells were transported had K_d values >10.0. Thus, a high percentage of cells of strains with low K_d values moved relatively freely through the soil, whereas a low percentage of cells of strains with high K_d values were transported at a significant rate under identical conditions.

Table 3. Adsorption coefficients of bacteria

Bacterium	K_d value (ml g^{-1}) Lower bound	Upper bound
Enterobacter IS2	4.5	5.5
Enterobacter IS1	0.0	0.0
Pseudomonas KL2	5.5	8.5
Arthrobacter Lula D	5.5	8.0
Pseudomonas DF2	0.85	0.9
Achromobacter Ben2	13	25
Pseudomonas Ben1	6.0	8.5
Bacillus CB2	18	36
Achromobacter DF1	7.5	11
Enterobacter KL1	3.5	4.5
Pseudomonas KL3	9.0	24
Enterobacter Tol2	29	145
Enterobacter Tol3	6.5	8.5
Pseudomonas Tol4	45	∞
Enterobacter Tol1	9.5	16
Pseudomonas CB1	26	150
Bacillus CU4519	30	410
Flavobacterium DF3	13	17
Bacillus CB3	12	24

The relationship between K_d and transport is especially striking if recovery is considered. Thus, for strains for which <50% of the cells were recovered, <1% of the cells were transported for all the 8 isolates having mean K_d values of >10.0. Similarly, >2% of the cells were transported for 8 of the 9 species with recoveries >35% and mean K_d values <10.0. Mobility is thus strongly dependent on both adsorption and viability.

The relationship between the percentage of the cells transported and the lower- but not the upper-bound K_d values was statistically significant. The F values for the regressions were 7.40 and 3.26, respectively.

Regressions indicated that recoveries were related to the K_d values ($P = 0.05$). When the lower-bound K_d values exceeded 20 or the upper-bound K_d values exceeded 100 (*Enterobacter* Tol2, *Pseudomonas* Tol4 and CB1 and *Bacillus* CU4519), 25% or less of the cells were recovered. This probably reflects the inability to detect a significant fraction of the sorbed cells in the procedure used for counting.

DISCUSSION

Macropore flow may be a major mechanism of bacterial transport in soils. Therefore, the use of undisturbed soil columns might have provided data on bacterial transport that would have particular relevance to circumstances prevalent in the field (Smith *et al.*, 1985). However, because the geometry of macropores in the field may change frequently due to wetting, drying, freezing, thawing or the burrowing of invertebrate animals, movement of bacteria through the soil matrix may be necessary for biodegradative microorganisms to reach much of the chemical that is well dispersed in the soil. Columns of homogeneous soil were used to minimize uncontrolled, preferential movement of bacteria through macropores and thus to permit a definition of such factors as mechanical filtration and adsorption that control the movement of bacteria through the soil matrix itself. In the development of the procedure for determination of mobility, a loamy soil was selected to avoid the extremes of limited bacterial sorption and relatively free movement in sandy soils on the one hand and the restricted movement resulting from extensive mechanical filtration, as well as extensive sorption, in fine-textured soils on the other hand. Had a sandy or a fine-textured soil been used, the ability of the procedure to detect small differences in bacterial mobilities might have been reduced. Furthermore, species that were found to be extensively transported in a disturbed soil lacking a network of macropores would likely move even more readily in a natural, non-disturbed soil with significant macroporosity.

Saturated soil with a constant head of water was used to mimic bacterial movement under conditions of saturated flow. This permitted the occurrence of mass transport of the cells in the sufficiently large pores of the homogeneous soil. Bitton *et al.* (1974) showed that movement of bacteria through soil columns stopped when the water content was at or below field capacity, and Madsen and Alexander (1982) demonstrated that movement of bacteria through soil was not detectable in the absence of a transporting agent such as water.

The procedure here described has several advantages for testing bacterial mobility in the soil matrix. Spurious data on mobility resulting from bacteria moving at the interface between the soil and the column wall are avoided through the use of a relatively wide column. Macropores through which bacteria could move preferentially were eliminated by grinding, sieving the soil prior to preparing the column and uniform packing to a fixed bulk density. The slow wetting of the column from the bottom prevented the formation of preferential paths during saturation of the column with water. Bacterial death from predation or parasitism was avoided because sterile soil was used. Increases in cell numbers arising from growth and decreases associated with starvation were prevented by performing the tests of transport at 2–5 C. Furthermore, the marked differences in mobility among the isolates suggest that the proposed procedure does indeed distinguish among bacteria with different capacities for movement.

For inoculation of the surface of the soil with bacteria that can degrade organic pollutants to lead to the destruction of those compounds at some underground site, some of the added cells must move through the soil to the below ground zone of contamination. Evidence exists, however, that introduced organisms may fail because they are not transported to the sites containing the pollutant (Goldstein *et al.*, 1985). In this context, it is worth noting that many of the carefully controlled experiments in which inoculation resulted in biodegradation required transport to soil depths of only 10 cm (Barles *et al.*, 1979; Edgehill and Finn, 1983; McClure, 1972). Measurements such as those made in our study will enable extrapolation of the potential penetrability of the bacteria to considerably greater depths. The data show a significant inverse correlation between the mobility of the bacteria and the lower bound K_d values. There is good reason why the lower bound of K_d correlates better with transport than the higher bound. Adsorption on clay particles equal in size to or smaller than the cells (this adsorption being included in the upper bound but not in the lower bound) may not affect mobility as much as adsorption on bigger particles. Bacteria adsorbed on particles smaller than the cells may move to some extent together with the particles on which they are adsorbed. Such adsorption, at times, may even enhance the transport because it retards adsorption on bigger particles. Thus, the lower bound, which includes only adsorption on particles larger than the cells, is likely to correlate (inversely) better than the upper bound with mobility. Mechanical filtration and adsorption can effectively retard bacterial transport. If adsorption is weak, the transport of cells through the soil matrix may be controlled by other factors, such as mechanical filtration. We have found that bacterial transport was strongly correlated with cell size ($P = 0.01$) (Gannon *et al.*, 1991), suggesting the importance of mechanical filtration. When K_d is high (e.g. >10), mobility should be low even for small bacteria provided that mechanical filtration is limited by large pore sizes. Yet for bacteria with low K_d values (e.g. <10), cell size should be critical. Adsorption and recovery were also inversely related. It has also been found that a significant correlation did not exist between mobility and hydrophobicity, net surface charge and capsule formation of these bacteria (Gannon *et al.*, 1991).

The present findings suggest that it should be possible to obtain bacteria that have both the capacity to biodegrade unwanted organic compounds and the ability to move through earth materials to sites containing such compounds. By selecting more mobile bacteria, the physical constraint to bioremediation with inoculated bacteria may be overcome, and species able to destroy pollutants may move to the sites of pollution.

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